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Bioremediation of Petrochemical Wastewater Containing BTEX Compounds by a New Immobilized Bacterium *Comamonas* sp. JB in Magnetic Gellan Gum

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Abstract In this study, we investigated the bioremediation of petrochemical wastewater containing BTEX compounds by immobilized *Comamonas* sp. JB cells. Three kinds of magnetic nanoparticles were evaluated as immobilization supports for strain JB. After comparison with Fe_3O_4 and *a*-Fe_2O_3 nanoparticles, *r*-Fe_2O_3 nanoparticle was selected as the optimal immobilization support. The highest biodegradation activity of *r*-Fe_2O_3-magnetically immobilized cells was obtained when the concentration of *r*-Fe_2O_3 nanoparticle was 120 mg L^{-1}. Additionally, the recycling experiments demonstrated that the degradation activity of *r*-Fe_2O_3-magnetically immobilized cells was still high and led to less toxicity than untreated wastewater during the eight recycles. qPCR suggested the concentration of strain JB in *r*-Fe_2O_3-magnetically immobilized cells was evidently increased after eight cycles of degradation experiments. These results supported developing efficient biocatalysts using *r*-Fe_2O_3-magnetically immobilized cells and provided a promising technique for improving biocatalysts used in the bioremediation of not only petrochemical wastewater but also other hazardous wastewater.

Keywords BTEX compounds · *Comamonas* sp. JB · Immobilization · Magnetic nanoparticles · Petrochemical wastewater

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Introduction

BTEX compounds (benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylenes) are an important family of aromatic hydrocarbons that are components of petroleum, and its products such as gasoline and diesel fuel are widely used in industrial syntheses [1–3]. Meantime, because of the application of petroleum and its products in industrial processes, BTEX compounds are frequently found as the major organic pollutants in petrochemical, coking, and other industrial wastewater [4]. As it is well known, BTEX compounds possess toxic to humans and are confirmed or suspected carcinogens. Thus, the Environmental Protection Agency classifies them as priority pollutants, making their removal from polluted environments critical [5]. Therefore, it is necessary to establish effective methods to clean up BTEX compounds to protect the environment [6].

Many researchers have focused their studies on the isolation and identification of BTEX compounds-degrading microorganisms, such as *Pseudomonas*, *Pseudoxanthomonas*, *Burkholderia*, *Sphingomonas*, *Thauera*, *Dechloromonas*, *Rhodococcus*, *Janibacter*, and *Acinetobacter* [6–12]. Nevertheless, current studies are mostly focused on the metabolism pathways of BTEX compounds, as well as the genes and enzymes involved, and rarely on the development of an immobilization method for bioremediation [10, 13, 14]. The use of immobilized microorganisms rather than free cells in biotransformation is advantageous to enhance the stability of the biocatalyst and to facilitate its recovery and reuse [15]. These advantages have encouraged researchers to investigate the application of immobilized cells in the biodegradation of toxic compounds, such as phenol, pyridine, carbazole, and dibenzothiophene [16–19]. However, mass transfer limitation involved in substrate diffusion to the reaction system is still the major drawback in the application of an entrapment technique [20]. Recently, nanoparticles that represent a new generation of environmental remediation technologies have been used in the studies of immobilized microbial cells, which could reduce the mass transfer resistance of traditional immobilization processes, especially for magnetic nanoparticles [20–23]. Thus, it is necessary and significant to attempt to remove BTEX by immobilized BTEX degraders with magnetic nanoparticles.

In this study, the bioremediation of BTEX by a new immobilized bacterium *Comamonas* sp. JB in magnetic gellan gum was investigated. The selection of magnetic nanoparticles and degradation of BTEX compounds by magnetically immobilized cells of strain JB were studied. The qPCR assays were also investigated. Meantime, ecotoxicological assessment of the treated effluent was also carried out. In addition, the recycling of *r*-Fe_2O_3-magnetically immobilized cells and nonmagnetically immobilized cells coupling with activated zeolite on the treatment of petrochemical wastewater containing BTEX compounds was also tested.

Materials and Methods

Chemicals

Benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylenes were purchased from J&K Scientific Ltd. (China). Fe_3O_4 nanoparticle (diameter <20 nm, 98 %), *a*-Fe_2O_3 nanoparticle (diameter <20 nm, 99.9 %), and *r*-Fe_2O_3 nanoparticle (diameter <20 nm, 99.9 %) were purchased from DKnano Scientific Ltd. (China). Activated zeolite as the ammonium exchangers was

obtained from Zhejiang Shengshi Mining Industry Co., Ltd (China). All other commercially available chemicals were of analytical grade.

Bacterial Strain and Cultivation Conditions

Comamonas sp. JB was routinely grown in mineral salt medium (MSM), which contained KH_2PO_4 3.7 g L^{-1}, $K_2HPO_4{\cdot}3H_2O$ 5.2 g L^{-1}, NH_4Cl 2.0 g L^{-1}, Na_2SO_4 1.0 g L^{-1}, $MgSO_4$ 0.1 g L^{-1}, and 1 mL L^{-1} of trace metal solution as previous described [24]. Yeast extract (40 mg L^{-1}) was added to the MSM. Benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylene were dissolved in dimethyl sulfoxide and added to the MSM at a suitable concentration. All cultures or cell suspensions were incubated at 30 °C on a reciprocal shaker at 150 rpm. Cell suspensions of BTEX-grown JB were prepared separately by centrifugating the cultures in late exponential phase at 10,000×*g* for 5 min, washing cell pallets twice with MSM, and resuspending cells in MSM.

Preparation of Gel Beads and Immobilized Cells

Immobilization of cells was performed using a method as described previously [20]. The gellan gum (1 % wt/vol) and cell suspension of strain JB with a turbidity at 660 nm of 2.5 were mixed at ratio of cell wet weight to dry polymers powder of 3 (wt/wt). Nonmagnetically immobilized cells were formed by extruding the mixture through a syringe into 0.2 M $CaCl_2$ and letting it solidify for 2 h. For preparing magnetically immobilized cells, 80 mg L^{-1} of Fe_3O_4 nanoparticle, *a*-Fe_2O_3 nanoparticle, and *r*-Fe_2O_3 nanoparticle suspension were added to the abovementioned mixture of gellan gel and cell suspension, and the procedure was the same as that for nonmagnetically immobilized cells. Nonmagnetically immobilized inactive cells and magnetically immobilized inactive cells were prepared as described above.

Petrochemical Wastewater Treatment by Immobilization Cells

The petrochemical wastewater used in this study was from the petrochemical wastewater treatment plant (WWTP) located in northeast China. The raw wastewater quality is shown in Table S1. The raw wastewater was diluted 1:1 (*v*/*v*) in deionized water, and phenol and BTEX compounds were added to give prominence to degradation; the final concentrations of them are shown in Table S1. This high phenol- and BTEX-concentrated petrochemical wastewater was used in the following study. Study on the degradation of this petrochemical wastewater by three magnetically immobilized cells, nonmagnetically immobilized cells, and free cells of strain JB coupling with activated zeolite were carried out. The concentration of *r*-Fe_2O_3 nanoparticle (40 to 240 mg L^{-1}) in magnetically immobilized cells on degradation of the petrochemical wastewater was studied. In the recycling experiments, after each biodegradation batch, *r*-Fe_2O_3-magnetically immobilized cells were collected and then were washed once with MSM. After the MSM was drained, 50 mL of petrochemical wastewater containing BTEX were added to repeat the cycle. Samples were taken at intervals to monitor the concentrations of BTEX and the acute toxicity of effluent and influent samples were also tested by Microtox bioassays as described below. All experiments were performed in triplicate.

Quantitative Real-Time PCR Assays

The concentration of strain JB in nonmagnetically immobilized cells, *a*-Fe_2O_3-magnetically immobilized cells, Fe_3O_4-magnetically immobilized cells, and *r*-Fe_2O_3-magnetically immobilized cells was selected for qPCR assays, which were conducted in triplicate using PCR Thermal Cycler Dice Real Time System (TaKaRa, China) with the primer set 16sFn (5′-TGGCAGATTAGGTAGTTGGTGG-3′) and 16sRn (5′-CAAAAGCAGTTTACAACCCG AG-3′). The qPCR mixture (25 μL^{-1}) contained 12.5 μL^{-1} SYBR Premix Ex Taq (TaKaRa, China), 1 μL^{-1} of each primer (10 μM), and 2 μL template DNA. The thermal profile included 30 s of initial denaturation at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The amplicons were visualized and check by electrophoresis on agarose gel (1.5 %, wt vol^{-1}).

Analytical Methods

After each batch of biodegradation, the samples were extracted with two volume of methylene chloride for at least 1 h by inversion. The concentrations of BTEX were analyzed by gas chromatography with an HP-5 capillary column (Agilent Technologies, 6890 N) as the previously described [6]. The gas chromatography oven was programmed to increase from 60 °C (held for 1 min) to 220 °C at 10 °C min^{-1}, after which 220 °C was held for 3 min. The gas flow to the detector contained H_2 (40 mL min^{-1}) and synthetic air (450 mL min^{-1}), the detector temperature was 300 °C, the injection port temperature was 250 °C, and the 1 μL samples were loaded with an auto sampler with a split mode (5:1). The profiles of *m*-xylene and *p*-xylene mirrored each other, because they had the same retention time on the gas chromatography analysis chromatogram, thus the concentration of *m*/*p*-xylene was calculated by dividing the mixture concentrations of *m*-xylene and *p*-xylene by 2 [6]. Phenol concentration was analyzed using high-performance liquid chromatography (HPLC) system (Shimadzu LC20A; Thermo Hypersil ODS-2 column, 5 μm, 250×4.6 mm) as previously described [16]. NH_3-N concentration was obtained via a Nessler's reaction using UV/Vis spectrophotometer [15]. The acute toxicity of effluent and influent samples was assessed by Microtox bioassays using the luminescent bacteria *Vibrio fischeri* (NRRL B-11177) as the previously described [16]. The morphology of cells immobilized in gel beads was determined using a scanning electron microscope (SEM) (S-570; Hitachi, Japan).

Results and Discussion

Selection of Magnetic Nanoparticles for Immobilization

In this study, three kinds of magnetic nanoparticles (*a*-Fe_2O_3, *r*-Fe_2O_3, and Fe_3O_4 nanoparticles) were evaluated as the magnetic nanoparticle for the immobilization of strain JB in gellan gum. The biodegradation of petrochemical wastewater containing phenol and BTEX compounds was conducted by free cells, nonmagnetically immobilized cells, and magnetically immobilized cells (*a*-Fe_2O_3, *r*-Fe_2O_3, and Fe_3O_4 nanoparticles) coupling with activated zeolite, respectively. As shown in Fig. 1a, phenol and all BTEX compounds were completely consumed within 8 to 32 h by free cells. The activity of nonmagnetically immobilized cells was lower than that by free cells; phenol and all BTEX compounds were completely consumed within 12 to 36 h by nonmagnetically immobilized cells (Fig. 1b). The SEM image of

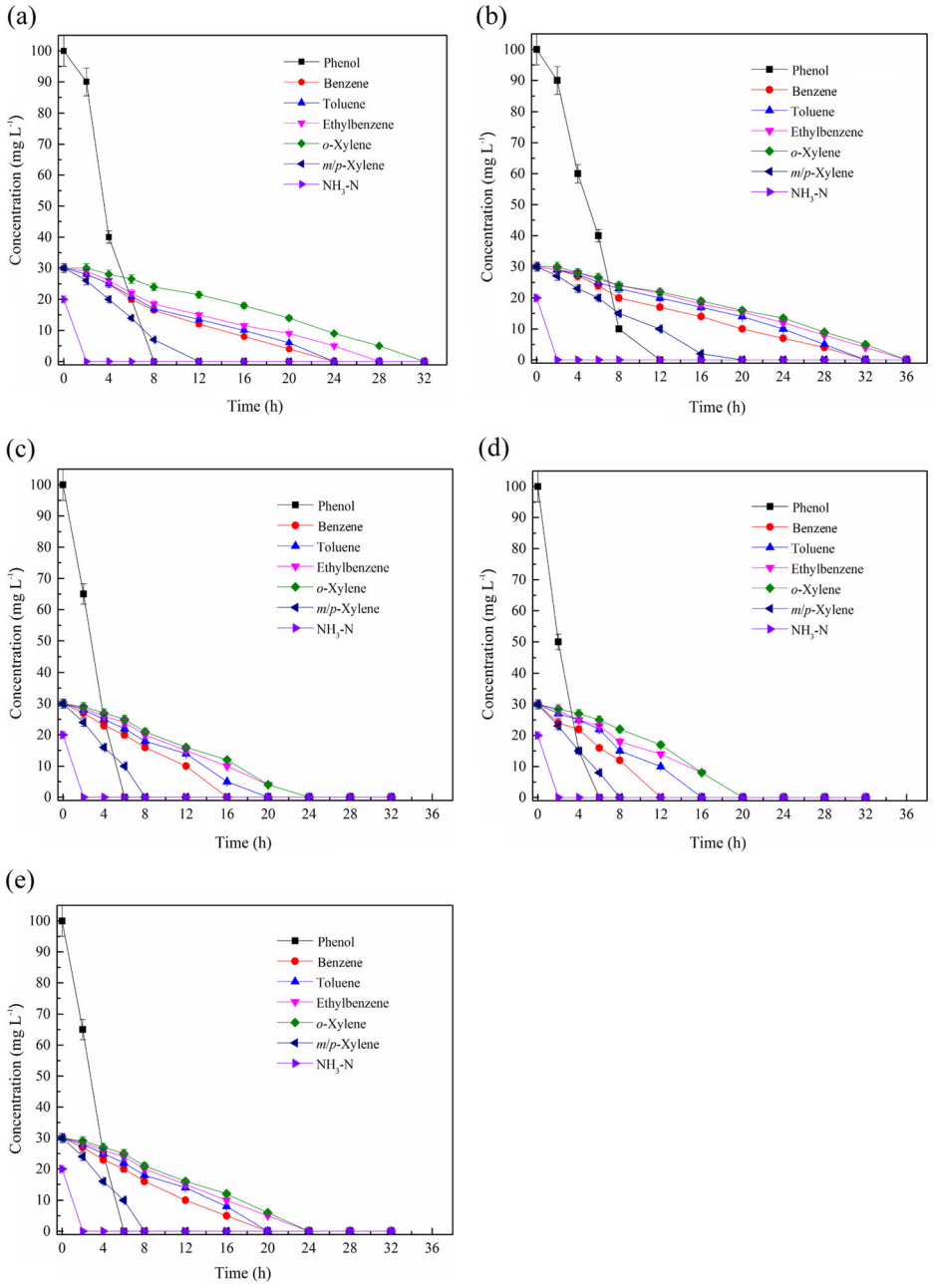

Fig. 1 Biodegradation of petrochemical wastewater containing phenol, benzene, toluene, ethylbenzene, *o*-xylene, *m*/*p*-xylene, and NH_3-N by free cells (**a**), nonmagnetically immobilized cells (**b**), magnetically immobilized cells (*a*-Fe_2O_3, **c**), magnetically immobilized cells (*r*-Fe_2O_3, **d**), and magnetically immobilized cells (Fe_3O_4, **e**)

nonmagnetically immobilized cells is shown in Fig. S1a. It indicated that strain JB cells could be clearly observed, and the sheets of gellan gum matrix were tightly bound together, which

may be resulted in impeding of the mass transfer of substrate from the environment to the central reaction site [20]. Additionally, no decrease of phenol and BTEX compounds content was observed when nonmagnetically immobilized inactive cells and gellan gel beads without cells served as biocatalysts, which confirmed that the removal of phenol and BTEX compounds was due to biodegradation by strain JB (data not shown).

In contrast, high biodegradation activities for phenol and BTEX compounds were obtained when magnetically immobilized cells served as the biocatalyst (Fig. 1c–e). Among these three magnetic nanoparticles, the highest biodegradation activities for phenol, benzene, toluene, ethylbenzene, *o*-xylene, and *m*/*p*-xylene were presented by *r*-Fe_2O_3-magnetically immobilized cells, 100 mg L^{-1} phenol could be degraded completely in 6 h, 30 mg L^{-1} and benzene, toluene, ethylbenzene, *o*-xylene, and *m*/*p*-xylene could be degraded completely within 12, 16, 20, 20, and 8, respectively. Magnetically immobilized cells with *a*-Fe_2O_3 and Fe_3O_4 nanoparticle showed slightly lower biodegradation activities for phenol and all the BTEX compounds than that with *r*-Fe_2O_3 nanoparticle, and phenol and all the BTEX compounds could be degraded completely within 6 to 24 h. Meanwhile, the SEM images of magnetically immobilized cells indicated that the sheets of gellan gum matrix were loosely bound together, and many pores existed between the sheets of gellan gum matrix in magnetic gellan gel beads, especially for immobilization with *r*-Fe_2O_3 nanoparticle (Fig. S1 b–d). It confirmed that the existence of nanoparticle could reduce or eliminate mass transfer problems to improve the biodegradation activities of magnetically immobilized cells. Therefore, *r*-Fe_2O_3 nanoparticle was chosen as the most suitable magnetic nanoparticle in the subsequent experiments. Because of the ion exchange adsorption of activated zeolite, all the NH_3-N could be completely removed within 2 h.

Bioremediation of Petrochemical Wastewater by *r*-Fe_2O_3-Magnetically Immobilized Cells

The effects of different concentrations of *r*-Fe_2O_3 nanoparticle (40, 80, 120, and 240 mg L^{-1}) on the activity of immobilized cells were studied. Figure 2 shows that the biodegradation rate was highest at an *r*-Fe_2O_3 nanoparticle concentration of 120 mg L^{-1}, 100 mg L^{-1} phenol could be degraded completely in 4 h, and 30 mg L^{-1} benzene, toluene, ethylbenzene, *o*-xylene, and *m*/*p*-xylene could be degraded completely within 8, 12, 16, 16, and 6, respectively. The equivalent amount of phenol, benzene, toluene, ethylbenzene, *o*-xylene, and *m*/*p*-xylene at *r*-Fe_2O_3 nanoparticle concentration of 80 mg L^{-1} could be degraded completely within 6, 12, 16, 20, 20, and 8, respectively. While the biodegradation rate was lower at an *r*-Fe_2O_3 nanoparticle concentration of 40 and 240 mg L^{-1}, phenol, benzene, toluene, ethylbenzene, *o*-xylene, and *m*/*p*-xylene could be degraded completely within 6, 16, 20, 24, 28, and 12 h, respectively. These results revealed that the biodegradation activity of the immobilized strain JB cells was significantly enhanced by adding 120 mg L^{-1} *r*-Fe_2O_3 nanoparticle, which may be due to the reduction or elimination of mass transfer problems.

Reuse of *r*-Fe_2O_3-Magnetically Immobilized Cells in the Bioremediation of Petrochemical Wastewater

In an industrial bioremediation process, the recycling of the biocatalysts could be an important factor that determines the effectiveness of degradation over time. The activities of *r*-Fe_2O_3-magnetically immobilized cells and nonmagnetically immobilized cells coupling with

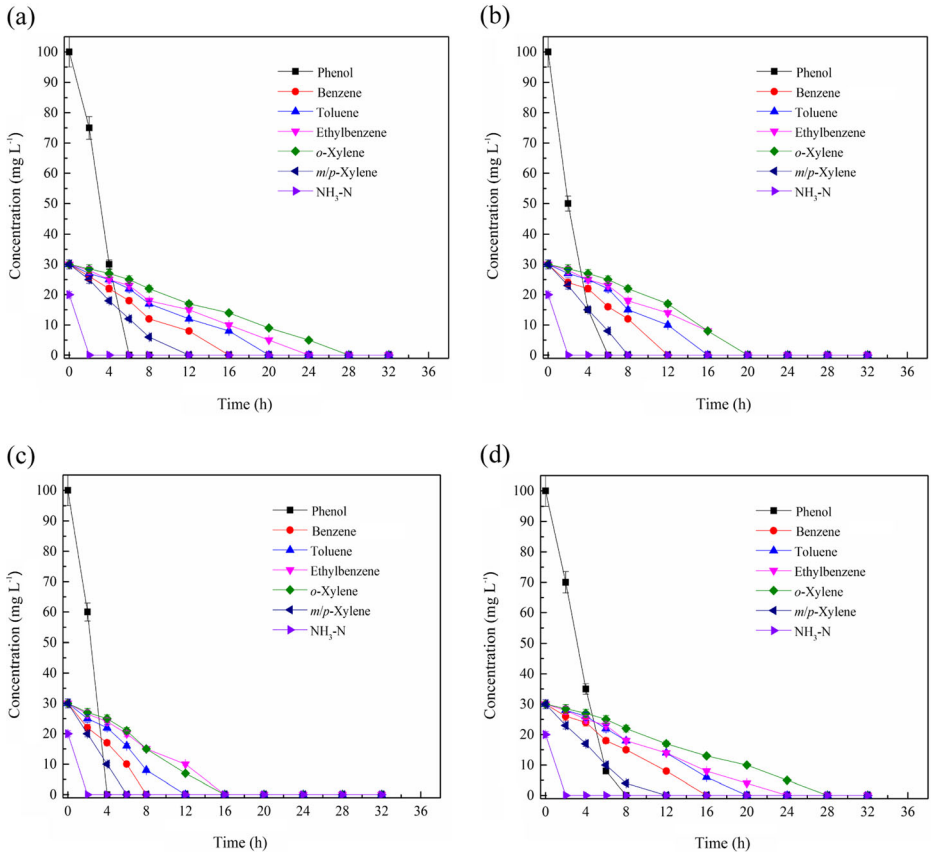

Fig. 2 Biodegradation of petrochemical wastewater containing phenol, benzene, toluene, ethylbenzene, *o*-xylene, *m*/*p*-xylene, and NH_3-N by *r*-Fe_2O_3-magnetically immobilized cells at different concentrations of *r*-Fe_2O_3 nanoparticle. **a** *r*-Fe_2O_3-magnetically immobilized cells at an *r*-Fe_2O_3 nanoparticle concentration of 40 mg L^{-1}, **b** *r*-Fe_2O_3-magnetically immobilized cells at an *r*-Fe_2O_3 nanoparticle concentration of 80 mg L^{-1}, **c** *r*-Fe_2O_3-magnetically immobilized cells at an *r*-Fe_2O_3 nanoparticle concentration of 120 mg L^{-1}, and **d** *r*-Fe_2O_3-magnetically immobilized cells at an *r*-Fe_2O_3 nanoparticle concentration of 240 mg L^{-1}

activated zeolite in the bioremediation of petrochemical wastewater were tested repeatedly. As shown in Fig. S2a and S3, from the first to the fifth cycle, *r*-Fe_2O_3-magnetically immobilized cells exhibited high biodegradation activity, and all the compounds were completely consumed in 20 h; from the sixth to the eighth cycle, the same amount of all the compounds were completely consumed in only 16 h. In contrast, from the first to the third cycle, all the compounds were completely consumed in 36 h by nonmagnetically immobilized cells (Fig. S2b and Fig. S4); from the fourth to eighth cycle, the degradation rate of phenol and *m*/*p*-xylene was also 100 %, while the degradation rate of benzene, toluene, ethylbenzene, and *o*-xylene was decreased from 99 to 90 %, 98 to 84 %, 98 to 83 %, and 95 to 75 %, respectively. For the removal of NH_3-N, the NH_3-N removal rate was decreased from 100 to 30 % during initial three cycles, while the removal rates of NH_3-N were decreased from 30 to 0 % from the fourth to eighth cycle might be due to the saturated adsorption ability to NH_3-N by activated zeolite. Figure S1e is the SEM image of *r*-Fe_2O_3-magnetically immobilized cells after eight cycles of the biodegradation experiments. As shown in Fig. S1e, the sheets of gellan gum

matrix were also loosely bound together. Moreover, the number of pores that existed between the sheets of gellan gum matrix evidently increased, which could further reduce or eliminate of mass transfer problems to improve the biodegradation activities of magnetically immobilized cells after eight cycles of the biodegradation experiments. Similar results were obtained in previous studies on the degradation of carbazole by Fe_3O_4-magnetically immobilized cells [20]. All these results indicated that *r*-Fe_2O_3-magnetically immobilized cells should be a promising biocatalyst used in biodegradation of petrochemical wastewater.

Quantitative Real-Time PCR Assays

In order to investigate the concentration of strain JB in nonmagnetically immobilized cells and magnetically immobilized cells, qPCR was done using the 16s V3 genes from strain JB as the standard. As shown in Fig. S5, the concentrations of strain JB in *a*-Fe_2O_3-magnetically immobilized cells (sample 2), Fe_3O_4-magnetically immobilized cells (sample 3), and *r*-Fe_2O_3-magnetically immobilized cells (sample 4) after first cycle of degradation experiment were almost the same as those in nonmagnetically immobilized cells (sample 1). It confirmed that high biodegradation activities for phenol and BTEX compounds obtained by magnetically immobilized cells might be supported by the existence of nanoparticles, the loose binding of the sheets of gellan gum matrix, and the existence of many pores between the sheets of gellan gum matrix (Fig S1). The concentration of strain JB in nonmagnetically immobilized cells (sample 5) and *r*-Fe_2O_3-magnetically immobilized cells (sample 6) after eighth cycle of degradation experiment was also investigated, and the concentration of strain JB was 438982 and 510852 copies ng L^{-1}DNA, respectively. The concentration of strain JB in *r*-Fe_2O_3-magnetically immobilized cells after eighth cycle of degradation experiment was significantly increased, which indicated that high biodegradation activity for phenol and BTEX compounds might by be supported by the good growth of cells in the magnetic gellan gel bead. These results were also consistent with a previous report that the growth of cells in the magnetic gellan gel beads was considered to have enhanced the biodegradation activity for carbazole in the recycling experiments [20].

Toxicity Assessment (Microtox Test)

In this study, ecotoxicity estimation was conducted by using the Microtox test (bacterium *V. fischeri*) to determine the change in effluent toxicity during the bioremediation of petrochemical wastewater by *r*-Fe_2O_3-magnetically immobilized cells coupling with activated zeolite as previous described [15]. The influent contained high levels of phenol, benzene, toluene, ethylbenzene, *o*-xylene, and *m*/*p*-xylene and had high toxicity against strain *V. fischeri* as indicated by IR value that exceeded 96 % (Fig. 3). As shown in Fig. 3a, from the first to the second cycle, the IR value of effluent was to be 25 % (moderate toxicity) after treatment by *r*-Fe_2O_3-magnetically immobilized cells coupling with activated zeolite; from the third to the eighth cycle, the IR value of effluent was increased slightly (from 28 to 32 %). In contrast, from the first to the second cycle, the IR value of effluent was to be 35 % (moderate toxicity) after treatment by nonmagnetically immobilized cells coupling with activated zeolite and from the third to the eighth cycle, the IR value of effluent was increased significantly (from 40 to 57 %). These results further confirmed that *r*-Fe_2O_3-magnetically immobilized cells coupling with activated zeolite exhibited higher biodegradation activity on the

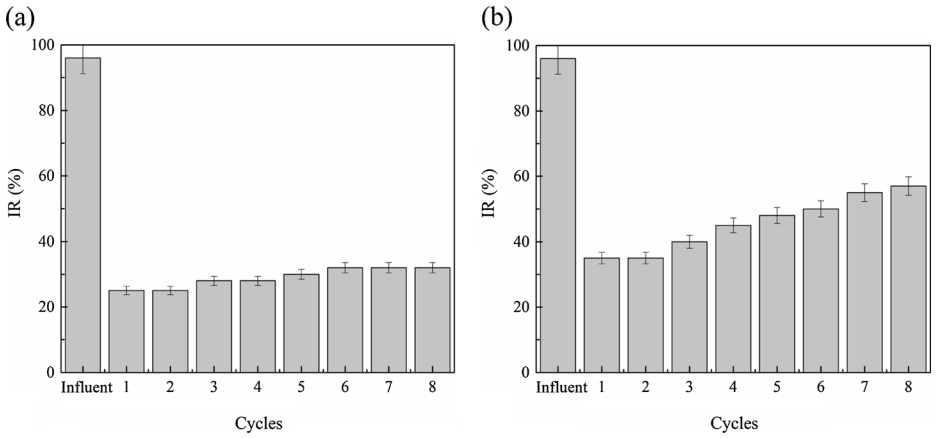

Fig. 3 Ecotoxicology assessment of the treated effluent with the reuse of *r*-Fe_2O_3-magnetically (**a**) and nonmagnetically (**b**) immobilized cells by bacterium *V. fischeri*

petrochemical wastewater than that by nonmagnetically immobilized cells coupling with activated zeolite and could led to less toxicity than the untreated petrochemical wastewater.

Conclusions

In this study, the bioremediation of petrochemical wastewater by *r*-Fe_2O_3-magnetically immobilized cells coupling with activated zeolite was investigated. *r*-Fe_2O_3 nanoparticle was chosen as the most suitable magnetic nanoparticle, and the optimal concentration was 120 mg L^{-1}. The recycling experiments demonstrated that the degradation activity of *r*-Fe_2O_3-magnetically immobilized cells was still high after eight cycles. According to these findings, magnetically immobilized cells of strain JB should be a promising biocatalyst used in biodegradation of petrochemical wastewater.

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